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**Citation for published version:**

Williams, GOS, Euser, TG, Russell, PSJ & Jones, AC 2013, 'Spectrofluorimetry with attomole sensitivity in photonic crystal fibres', *Methods and Applications in Fluorescence*, vol. 1, no. 1, 015003.  
<https://doi.org/10.1088/2050-6120/1/1/015003>

**Digital Object Identifier (DOI):**

[10.1088/2050-6120/1/1/015003](https://doi.org/10.1088/2050-6120/1/1/015003)

**Link:**

[Link to publication record in Edinburgh Research Explorer](#)

**Document Version:**

Peer reviewed version

**Published In:**

Methods and Applications in Fluorescence

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Cite as:

Williams, G. O. S., Euser, T. G., Russell, P. S. J., & Jones, A. C. (2013). Spectrofluorimetry with attomole sensitivity in photonic crystal fibres. *Methods and Applications in Fluorescence*, 1(1), [015003].

Manuscript received: 05/11/2012; Accepted: 14/12/2012; Article published: 28/01/2013

## Spectrofluorimetry with Attomole Sensitivity in Photonic Crystal Fibres\*\*

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<sup>[\*\*]</sup>This work forms part of a project funded by the Koerber Foundation. G.O.S.W. and A.C.J. also acknowledge the EPSRC for funding.

### Supporting information:

Online supplementary data available from <http://iopscience.iop.org/2050-6120/1/1/015003/media>

## Abstract

We report the use of photonic crystal fibres (PCF) as spectrofluorimetric systems in which sample solutions are excited within the microstructure of the fibre. The use of intra-fibre excitation has several advantages that combine to enable highly sensitive measurements of fluorescence spectra and lifetimes: long path-lengths are achieved by the efficient guidance of the fundamental mode; sample volumes contained within the micron-scale structure are very small, only a few nanolitres per cm of path; collection and guidance of the emitted fluorescence is efficient and the fluorescence lifetime is unperturbed. Fluorophores in bulk solution can be studied in hollow-core PCF, whereas the use of PCF with a suspended, solid core enables selective excitation of molecules in close proximity to the silica surface, through interaction with the evanescent field. We demonstrate the measurement of fluorescence spectra and fluorescence lifetimes in each of these excitation regimes and report the detection of attomole quantities of fluorescein.

## Introduction

Fluorescence spectroscopy has become widely used across many fields in the physical and biological sciences, with increasing use of techniques that exploit the ability to achieve (under carefully controlled conditions) detection at the ultimate limit of a single molecule.<sup>1,2</sup> However, quantitative fluorimetric measurement of fluorophores at low concentrations, free in bulk solution, or on surfaces, under practical laboratory conditions, remains a challenge.

Traditional methods of studying free fluorophores usually employ perpendicular excitation and detection of a solution contained in a cuvette. This leads to the relatively inefficient collection of fluorescence and the use of a sample volume that greatly exceeds the excitation/detection volume. In recent years there has been significant progress in presenting small sample volumes (nanolitre to picolitre) for measurement, in the form of microdroplets. This typically involves the use of a microfluidic droplet generator to produce a flow of aqueous droplets, suspended in a continuous oil phase, in a microfluidic channel, together with confocal fluorescence microscopy to enable excitation and detection of the droplets with micrometer resolution.<sup>3</sup> This approach is attractive for high throughput, fluorescence-based assays in which it is only necessary to detect the fluorescence intensity of a droplet, but the short residence time, as the droplet flows through the interrogation volume, is disadvantageous for the measurement of fluorescence spectra or lifetimes. Fluorescence lifetime imaging microscopy (FLIM) of microdroplets has been achieved, but adds further complexity to the experimental system. Bennet *et al.*<sup>4</sup> reported FLIM of a single microdroplet, using optical tweezers to trap the droplet in the microfluidic flow, while Solvas *et al.*<sup>5</sup> acquired decay data from several thousand continuously flowing droplets, using confocal FLIM, followed by burst analysis to discriminate between droplet fluorescence and background fluorescence from the continuous oil phase.

Photonic crystal fibre<sup>6</sup> (PCF) offers an alternative approach in which the guidance of light through nanolitres of static solution contained within the fibre microstructure facilitates wavelength-resolved or time-resolved fluorescence measurements. Moreover, it does not require special sample preparation and uses the same type of excitation sources and detection systems that are used in conventional cuvette-based spectrophotometry. Previously, we have demonstrated the application of PCF as an excitation and detection system for the study of photochemical systems<sup>7,8</sup> and we now show that it is a promising modality for ultrasensitive fluorescence measurements. Early reports of work using PCF for fluorescence measurements involved a similar system to conventional measurements, using a solid-core-PCF (SC-PCF) simply as a light delivery and collection device from a conventional sample cell.<sup>9,10,11</sup> This method takes advantage of the light transport properties of the fibre, allowing light to be guided easily to the sample, and exploits the fact that SC-PCFs can be designed to have a numerical aperture that is high compared with conventional optical fibres, enabling relatively efficient collection of fluorescence. If the modal properties of the excitation light are not of concern, large-core multi-mode SC-PCF can be used, further increasing collection efficiency. Several improvements over a single fibre system have been made leading to various applications. For example, Ma *et al.*<sup>7</sup> showed that the use of two or more fibres in a bundle, one conventional fibre to excite fluorescence and one or more SC-PCFs to collect the emission, greatly enhanced efficiency. The use of a SC-PCF in this regime for measuring fluorescence lifetimes was shown by Fujii *et al.*<sup>12</sup> who use a SC-PCF spliced to a conventional fibre to excite temperature-dependent fluorescence from quantum dots and collect it through the same fibre construct, allowing accurate temperature measurements from within the sample.

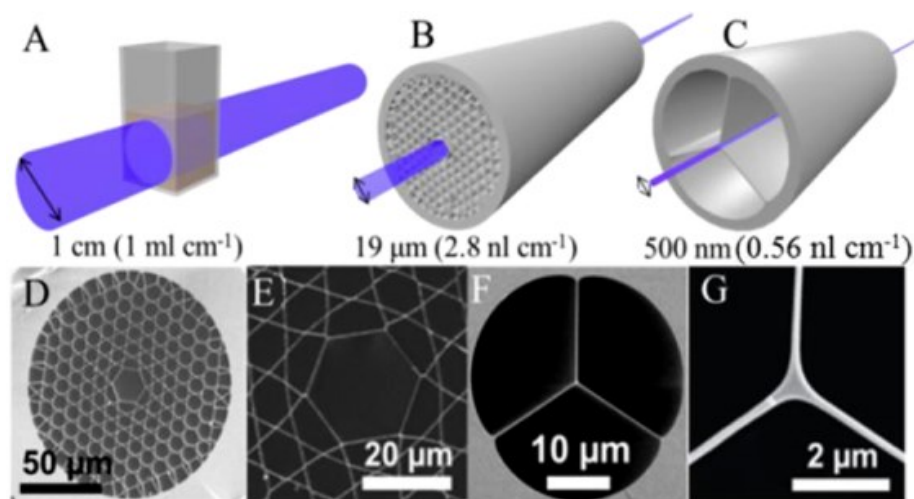
To take full advantage of the properties made available by the use of PCF, such as small internal volume and long path-lengths, one must excite and detect fluorescence from within the fibre itself. This should provide both efficient overlap of excitation light with the sample volume and efficient collection and quantitative detection of the emitted fluorescence within the fibre microstructure, potentially an ideal approach for monitoring fluorescence properties of molecules in solution. Measurements at very low concentrations should be achievable by exploiting the long excitation path-length, together with the possibility of excitation within the bulk solution or at the interface between the solution and the internal fibre surface. Both of these regimes were demonstrated by Konorov *et al.*<sup>13</sup> By varying the refractive index of the solution filling a hollow-core fibre, relative to the silica structure, the guidance properties of the fibre were modified. When using a low refractive index sample, light was guided in the silica construct allowing excitation of molecules close to the silica surface by the evanescent field. Using a higher refractive index sample, light was guided in the solution allowing bulk excitation. This showed the potential of PCF as a vessel for the excitation and collection of fluorescence spectra. However, as the index of the sample was used to change the guidance properties of the fibre, the guidance was always by total internal reflection within the liquid-filled core, not by the photonic band gap mechanism for which the fibre was designed, and, due to the relatively small index contrast, high losses would be incurred. A similar measurement by Jensen *et al.*<sup>14</sup>, using a PCF in the evanescent sensing regime and illuminating the whole fibre face, showed detection of Cy5-labelled DNA oligonucleotides at a

concentration of  $\sim 10$  nM (the number of fluorophores per oligonucleotides was not indicated). In this case, the ill-defined sample illumination prevented accurate estimation of the volume of the sample exposed to the excitation light, and hence the quantity of sample detected.

Smolka *et al.*<sup>15</sup> have shown the use of a hollow-core PCF (HC-PCF) for efficient excitation and collection of the fluorescence of bulk solution within the fibre core. In this measurement, the cladding holes of the 10-cm fibre were sealed by laser fusing, so that only the fibre core was filled, by capillary action. This, as in the Konorov experiments, produced a higher refractive index in the core than the cladding and therefore the fibre guided by total internal reflection within the liquid core, not by the photonic band gap, reducing efficiency. The same study used a SC-PCF to examine evanescent wave excitation. Limits of detection for the rhodamine 6G fluorophore of  $10^{-6}$  M in the SC-PCF and  $10^{-10}$  M in the HC-PCF were reported. The greatly increased sensitivity in the HC-PCF was attributed to the near 100% overlap of excitation light with sample, compared to  $\sim 1\%$  in the SC-PCF. The latter study is the closest approach to a true in-fibre bulk fluorescence measurement that has been reported, to our knowledge, but it still failed to exploit the enhanced guidance characteristics that are made possible through the use of PCF. We now demonstrate that rigorous exploitation of the unique guiding properties of PCF can achieve intra-fibre fluorescence detection limits superior to those reported previously and demonstrate, for the first time, intra-fibre fluorescence lifetime measurements in both HC-PCF and SC-PCF.

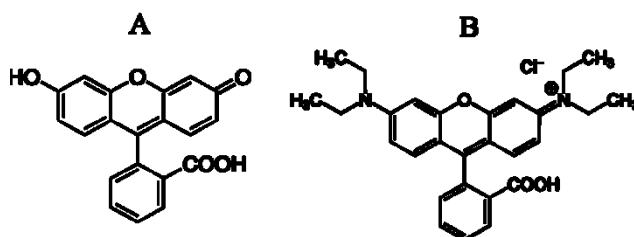
In Figure 1, we compare the sample volume that is excited typically in a 1-cm path-length cuvette (Fig 1A) with the excitation volume in the HC-PCF (Fig 1B) and the SC-PCF (Fig 1C) used in this study. The microscopic structures of the kagomé-type HC-PCF and the SC-PCF are shown in Figure 1(D to G). In the HC-PCF, the hollow core (diameter 19  $\mu\text{m}$ ) contains a sample volume of 2.8 nl per cm path-length and there is  $\sim 98\%$  overlap between the guided excitation beam and the sample in the fibre core.<sup>8</sup> In the SC-PCF, the excitation is guided through the 500-nm-diameter solid core and the solution in the surrounding hollow structure, 7 nl per cm path-length, is excited by the evanescent field. The overlap of the evanescent field with the sample volume is  $\sim 8\%$ ,<sup>16</sup> giving an excitation volume of  $0.56 \text{ nl cm}^{-1}$ .

The kagomé-type hollow core fibre used in these experiments (Figure 1(D-E)) exhibits low losses of  $\sim 1 \text{ dB m}^{-1}$  over a large spectral range. (We note that the guidance mechanism in this lattice structure is not completely understood and cannot be attributed to a photonic band-gap. Guidance of the fundamental mode in the hollow core is believed to be achieved due to the low density of states of the cladding modes and their weak interaction with the guided fundamental mode. This type of fibre was used instead of the alternative ultra-low-loss ( $\sim 1 \text{ dB km}^{-1}$  best case<sup>17</sup>), band-gap guiding, HC-PCF fibres due to the increased spectral range that can be guided<sup>18</sup>). The broad transmission window of the kagomé fibre, from  $\sim 400 \text{ nm}$  to  $\sim 800 \text{ nm}$ , enables the guidance of the pump laser and the collection of the complete emission spectrum of the fluorophores. The SC-PCF (Fig 1(F-G)) also provides broadband guidance (through the solid core), from  $\sim 300 \text{ nm}$  to  $\sim 2 \mu\text{m}$ , with losses of  $\sim 0.1 \text{ dB m}^{-1}$ , with a wavelength-dependent evanescent field penetrating the sample.



**Figure 1.** Schematic comparison of the excitation geometries and excitation volumes in a conventional cuvette (A), a hollow-core PCF (B) and a suspended-core PCF (C). High-resolution scanning electron micrographs (SEM) of the kagomé HC-PCF, with a core diameter of 19  $\mu\text{m}$  (D and E), and the SC-PCF, with a core diameter of 500 nm (F and G).

Detection limits for both fibre types were established using fluorescein, a fluorophore which forms the basis of a wide range of fluorescent labels and probes used in biomolecular assays. To study fluorescence lifetime detection from within the fibres the rhodamine B fluorophore was used. The structures of the two fluorophores are depicted in Figure 2.



**Figure 2.** Structures of the fluorescein (A) and rhodamine B (B) fluorophores

The rate of non-radiative decay of rhodamine B is influenced by the ability of the alkylamino substituents to rotate relative to the aromatic molecular framework (internal rotation).<sup>19,20</sup> The effect of rotation of these groups from the planar to the twisted form is an increase in the rate of internal conversion, resulting in a decrease in the measured fluorescence lifetime (and the fluorescence quantum yield).<sup>21,22</sup> This allows the molecule to be used as a temperature probe, based on the thermally-induced decrease in the measured lifetime,

as shown by Mendels *et al.*<sup>23</sup>. Another consequence of this torsional influence is that immobilisation of the molecule on a surface, which restricts the internal rotation, increases the observed lifetime. This was demonstrated by Smith *et al.*<sup>24</sup> by adsorption of the fluorophore on colloidal silica, allowing the lifetime of the free and immobilised forms to be determined. A value of 3.65 ns was found for the lifetime of rhodamine B on the silica surface, compared with a lifetime of 2.1 ns in aqueous solution at 20 °C.<sup>20</sup> The difference in the lifetime between the solution-phase and surface-associated fluorophore is used in the present study to probe the location of the excited fluorophores with respect to the surface of the fibre core.

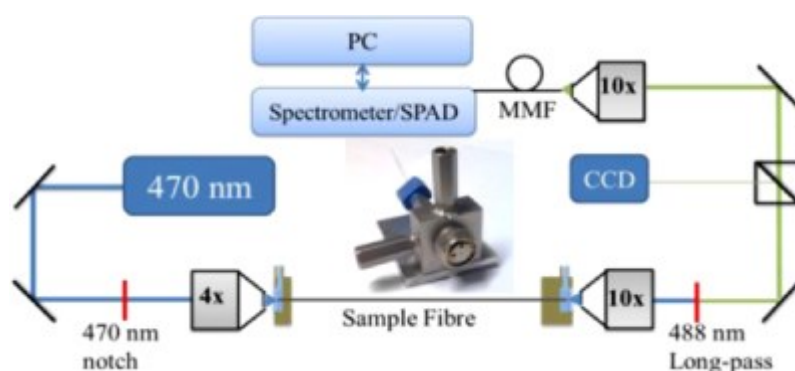
## Experimental

The photonic crystal fibres were fabricated in-house at the Max Planck Institute for the Science of Light. Fluorescein and rhodamine B were purchased from Sigma–Aldrich and used as received. Solutions were prepared using HPLC-grade water. The pH of fluorescein solutions was adjusted to a value of 7.5 to ensure that only the dianion form of the molecule was present<sup>25</sup>.

The experimental arrangement is shown in Figure 3. The excitation source was a picosecond pulsed 470-nm diode laser (Picoquant), the output of which was coupled into the sample fibre via a 470-nm notch filter. The spatial coupling of the excitation laser into the sample fibre was monitored using a CCD camera. Emitted fluorescence was collected and guided by the sample fibre and collimated at the output. The transmitted excitation was separated from the emission by a 488-nm long pass filter after the fibre output. For spectral measurements, emission was detected by a low-cost, miniature, fibre-coupled CCD grating spectrometer (Ocean Optics USB 2000+). For fluorescence lifetime measurements, the spectrometer was replaced by a fibre-coupled single-photon-counting avalanche diode detector (SPAD) and time-correlated single photon counting (TCSPC) system (Edinburgh Instruments). Decay curves were analyzed using a standard, iterative, non-linear least squares method, assuming a multiexponential decay function, where  $A_i$  is the fractional amplitude and  $\tau_i$  is the fluorescence lifetime of the  $i^{\text{th}}$  decay component.

Measurements were made using ~30 cm lengths of each type of PCF. Each end of the PCF was mounted in a custom-built liquid cell (Figure 3 insert), manufactured in-house, to allow straightforward introduction of the sample solution into the fibre, via a syringe pump, whilst maintaining optical alignment and minimising dead volume. (Although a syringe pump was used here for convenience, a controlled flow rate is not required and it would be perfectly satisfactory to use a manual syringe to introduce the solution). An optical window on the cell allows coupling of light into/out of the fibre. For the HC-PCF a 4x objective whose numerical aperture was matched to that of the fibre mode was used for in-coupling, as shown in Fig 3. Typical in-coupling efficiencies of approximately 80% were achieved; for the SC-PCF a 60x objective was used because of the small core size.

In the case of the HC-PCF, in which molecules in the bulk solution are excited, the fibre can be flushed out with solvent between samples and re-used. For the SC-PCF, in which molecules in close proximity to the surface of the solid core are excited, the retention of even a small concentration of surface-bound molecules could be problematic when changing the sample, so replacement of the fibre is advisable. This is a practical proposition since the fibre is manufactured by the km.



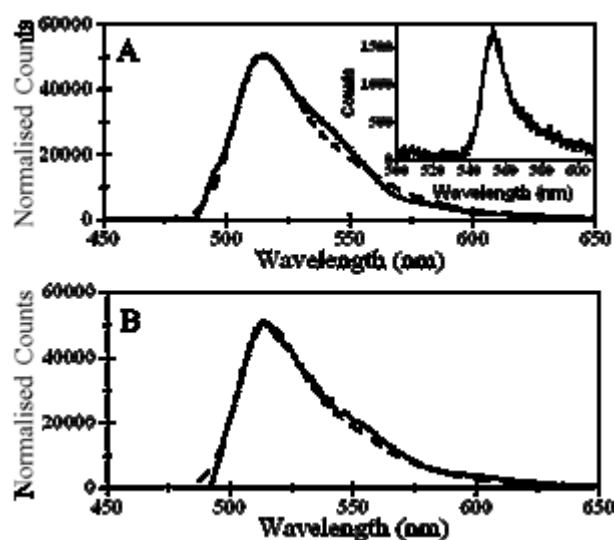
**Figure 3.** Schematic of the experimental setup for fluorescence excitation within a PCF. The 470-nm diode laser source is aligned through and coupled into the HC-PCF or SC-PCF containing the sample. A 470-nm notch filter is used to ensure that only the desired excitation light is coupled into the fibre. The emitted fluorescence collected by the fibre is filtered using a 488-nm long-pass filter to remove the excitation beam and is then conducted to the spectrometer (or SPAD) via a multimode fibre (MMF). The inset shows the liquid cell for introduction of sample solution into the fibre.

## Results and Discussion

### Fluorescence spectra

Fluorescence spectra recorded for fluorescein solutions of concentration  $10^{-8}$  M, in both HC-PCF and SC-PCF, are shown in Figure 4, in comparison with the spectrum measured for a  $10^{-6}$  M solution in a 1-cm cuvette. The spectral profiles of the intra-fibre fluorescence are in good agreement with the reference cuvette spectrum, apart from a small distortion of the long-wavelength edge in the vicinity of the water Raman band (around 550 nm). The Raman signal measured for a 30-cm length of water-filled HC-PCF is shown in the inset in Figure 4. The similarity of the spectra measured in the bulk solution in the HC-PCF and near the solution-silica interface (SC-PCF) is consistent with previous observations, using conventional evanescent-wave induced fluorescence spectroscopy, that the spectrum of fluorescein is unperturbed by proximity to the surface.

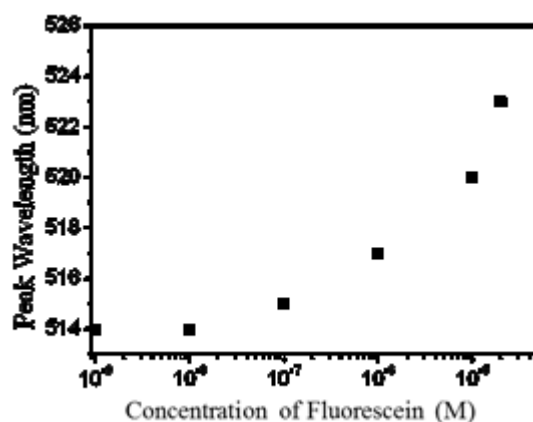




**Figure 4.** Fluorescence spectra for  $10^{-8}$  M aqueous solution of fluorescein in the HC-PCF(A) and the SC-PCF (B). In each case the spectrum measured for the intra-fibre solution (solid line) is compared with the spectrum measured in a cuvette (dashed line) The inset shows the Raman signal recorded for water in a 30-cm length of HC-PCF.

In the HC-PCF, the fluorescein emission may be subject to reabsorption as it passes through the long path-length of solution between excitation and detection, as a result of the overlap between the absorption and emission spectra. For the 30-cm path-length used here, this effect becomes evident as a shift in the wavelength of the emission maximum at concentrations above

$10^{-8}$  M. As shown in Fig 5, the emission maximum shifts from 514 nm to 523 nm as the concentration is increased from  $10^{-8}$  M to  $2 \times 10^{-5}$  M, as a result of increasing absorption of the shorter emission wavelengths.

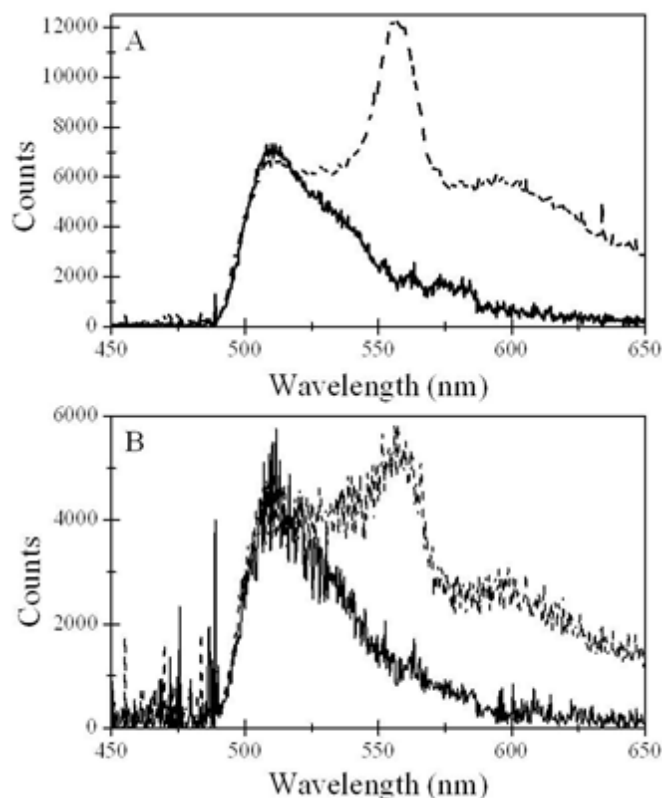


**Figure 5.** The effect of increasing concentration on the wavelength of the emission maximum of fluorescein in the HC-PCF.

The fluorescence spectrum recorded in the HC-PCF at a fluorescein concentration of  $2 \times 10^{-11}$  M, approaching the detection

limit, is shown in Fig 6A. At this very low concentration, the water Raman band is now the dominant feature in the spectrum, relative to the greatly reduced fluorescence signal. Background subtraction, using a pure water reference spectrum, allowed removal of the Raman band, recovering the correct spectral profile of fluorescein. At this concentration, the  $\sim 90$  nl of solution within the measurement volume of the fibre core contains only  $1.8 \times 10^{-18}$  moles (1.8 attomoles) of the fluorophore, about  $10^6$  molecules. On the basis of the signal-to-noise ratio of the corrected spectrum in Figure 6A, we estimate that the detection limit is about a factor of ten less than this, i.e. sub-attomole.

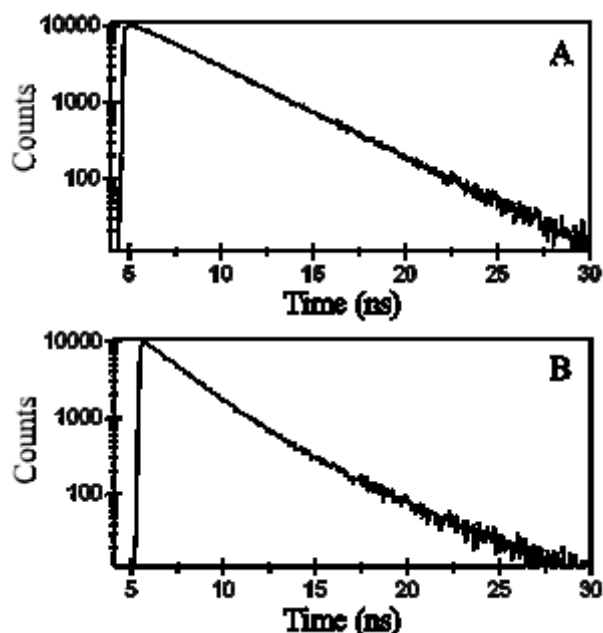
The detection sensitivity that can be achieved in the SC-PCF, using evanescent-wave excitation, is inevitably lower because of the relatively small light-sample overlap. The spectra measured for  $3 \times 10^{-10}$  M fluorescein in the SC-PCF, before and after water-background subtraction, are shown in Figure 6B. In this case,  $\sim 60$  attomoles ( $7 \text{ nl cm}^{-1}$ ) of fluorescein are contained in the fibre surrounding the core; however, the number of molecules that are excited by the evanescent wave is only about 1 attomole, similar to the number excited in the HC-PCF at  $2 \times 10^{-11}$  M.



**Figure 6.** Fluorescence spectra of fluorescein solution (A) in the HC-PCF at a concentration of  $2 \times 10^{-11}$  M and (B) in the SC-PCF at a concentration of  $3 \times 10^{-10}$  M. In each case, the spectrum is shown before (---) and after (—) subtraction of the water background.

## Fluorescence Lifetimes

The fluorescence decay measured for  $10^{-9}$  M rhodamine B, in each type of fibre, is shown in Figure 7.



**Figure 7.** Fluorescence decays measured for an aqueous solution of rhodamine B ( $10^{-9}$  M) in the SC-PCF (A) and the HC-PCF (B). The fitted decay functions are shown in Figure S1 of the Supporting Information.

In the SC-PCF, the measured decay (Fig 7A) is mono-exponential with a lifetime of  $3.64 (\pm 0.17)$  ns. This is in excellent agreement with the lifetime reported previously for rhodamine B adsorbed on colloidal silica<sup>21</sup> and confirms that, in the SC-PCF, molecules at the interface between the solution and the solid core are excited exclusively by the evanescent field.

In the HC-PCF (Fig 7B), a shorter decay time of  $2.12 (\pm 0.13)$  ns is observed, equivalent to that reported for rhodamine B in bulk solution at 20 °C. In fact, this decay shows slight deviation from mono-exponential behaviour. The quality of fit is significantly improved by the addition a second, minor decay component with lifetime of  $4.0 (\pm 1.4)$  ns and amplitude of 6%. There is a large uncertainty in the latter lifetime value because of the very small amplitude of this component, but it approximates to that of surface-adsorbed rhodamine B. Thus, there is slight heterogeneity of the emitting population in the HC-PCF; the vast majority (94%) of the fluorophores that are excited are in bulk solution, while only a very small fraction (6%) is subject to surface perturbation, despite the high surface-area-to-volume ratio of the hollow core, which is of the order of  $10^5 \text{ m}^{-1}$ . An increase in core diameter would serve to reduce the effect of surface perturbation, but would also reduce guidance efficiency at shorter wavelengths.

## Conclusion

We have shown that PCF can be used as the basis of a simple experimental system for highly efficient spectrofluorimetry of solutions contained within the fibre microstructure. It is straightforward to couple the PCF-based optofluidic sample system to a variety of commonly used excitation sources and detection systems. Here we have used a diode laser for excitation, but we have shown previously that LED or lamp sources can be coupled into the PCF.<sup>7,8</sup> Indeed, the PCF sample system could be coupled readily to a conventional fluorescence spectrometer in place of the standard cuvette.

In HC-PCF, by exploiting the near-perfect overlap of the excitation beam with solution within the fibre core, together with efficient guiding of the emitted fluorescence, wavelength-resolved detection of fluorescence from 90 nL of  $2 \times 10^{-11}$  M fluorescein was demonstrated. This equates to only 1.8 attomoles ( $\sim 10^6$  molecules) in the measurement volume. The detection limit is estimated to be factor of ten lower than this. This compares favourably with the previously reported detection limit of  $10^{-10}$  M for rhodamine 6G in a HC-PCF.<sup>12</sup> In SC-PCF, the measurement of evanescent-wave-induced fluorescence at sub-nanomolar fluorophore concentrations was achieved, much lower than the micromolar limit reported previously.<sup>12</sup>

Time-resolved measurements demonstrated, for the first time, the ability of PCF to preserve the fluorescence decay profile of fluorophores excited within the fibre, allowing accurate measurement of fluorescence lifetimes for both evanescent and bulk excitation. These measurements also confirmed that in HC-PCF excitation is confined to the bulk solution, whereas in SC-PCF molecules at the solution-solid interface are exclusively excited. We anticipate that the temperature-dependence of the rhodamine B lifetime could be exploited to good effect for intra-fibre thermometry in HC-PCF. This would be a useful adjunct to studies requiring appropriate temperature control, such as the trapping of microparticles or biological cells within such fibres which has been explored by Euser *et al.*<sup>26,27</sup>

The ability to measure evanescent-wave-induced fluorescence over long path-lengths in HC-PCF offers a significant advance in sensitivity over the conventional procedure, in which the emission is measured from a single excitation point at the interface between a silica prism<sup>28</sup> and bulk solution<sup>29</sup>. The latter approach uses, typically, concentrations of at least  $10^{-6}$  M and sample volumes of the order of 0.1 ml. An interesting application of evanescent-wave-induced fluorescence is in studying the effect of surface-adsorption on the conformational properties of biomolecules, such as oligonucleotides.<sup>30</sup> This is relevant to both biological function and biotechnological systems which frequently employ surface-immobilised molecules. The adoption of SC-PCF as a vehicle for such measurements could extend their scope to a wide range of biomolecules that may only be available in very small quantities.

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